The responses to flooding under elevated CO2 in Black Spruce (*Picea mariana*) and Tamarack (Larix laricina) seedlings

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EFFECTS OF CO_2 ELEVATION ON THE RESPONSES TO FLOODING IN BLACK SPRUCE (*P. MARIANA*) AND TAMARACK (*L. LARICINA*) *SEEDLINGS*

by

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This thesis was written in the manuscript style, and is meant to be read as two separate but related journal articles.

Abstract

Anthropogenic activities have been increasing the atmospheric CO_2 concentration globally and the increase is predicted to continue to rise in the future. Increased CO_2 has been shown to increase photosynthetic rates as well as water use efficiency in trees as well as induce changes in the global climatic conditions. Climate change is also expected to alter the world's hydrologic cycles, leading to increased flooding events in many regions. Prolonged flooding events can cause negative effects on even flood tolerant species such as *Picea mariana* and *Larix laricina,* because of the lack of soil aeration. The lack of air in the soil causes the roots to turn from aerobic root respiration to fermentation. Fermentation can lead to root tissue die back as a result of carbohydrate use inefficiency. The goal of this study was to determine if the benefits gained by increased CO_2 could overcome the problems caused by flooding. To test the effects of increased CO_2 and flooding on *P. mariana* and *L. laricina* changes in morphology and physiology were examined by exposing seedlings to double atmospheric CO_2 and a 28 day flood with a 35 day recovery. Overall no interactive effects on the morphology of either seedling species were found. There was only one significant interaction between CO_2 and flooding seen on the physiology of the seedlings; though no long lasting effects were observed. The flooding treatment, however, significantly reduced the quantum yield of Photosystem II and root respiration in both species and under both CO_2 concentrations. The Photosystem II function recovered 35 days after the termination of the flood treatment, but root respiration did not. The results suggest that the CO_2 elevation did not alleviate the flood injuries or enhance the subsequent recovery following the flood treatment. It is also found that the two species has similar tolerance to flooding despite that the flood treatment induced the development of adventitious roots in *L. laricina* but not in *P. mariana*.

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Chapter 1: General Introduction

Anthropogenic activities have caused global climate changes, including increasing global temperatures and changes in precipitations. The increases in the atmospheric CO_2 concentration in the past century have been considered as the primary culprit for the global changes (Diffenbaugh and Field 2013). However, rising atmospheric CO_2 has been shown to increase photosynthetic rates and plant productivity (Bazzaz et al. 1993, Drake et al. 1997, Curtis and Wang 1998, Anderson-Teixeira et al. 2013) as well as plant water-use efficiency (Keenan et al. 2013). Anthropogenic climate change is also expected to alter hydrological cycles globally, leading to increases in flooding in many areas (Huntington 2006). Wetlands in particular are expected to experience increased flooding seasonally with more intense floods predicted with further increases in atmospheric CO_2 and temperature (Huntington 2006, Kreuzwieser and Rennenberg 2014). Wetlands make up nearly 12% of the landmass in Canada (Yang and Yeh 2007). The two dominant tree species growing on these wetlands are *P. mariana* and *L. laricinia* (Uchytil 1991, Ontario Ministry of Natural Resources 2013a). These tree species are flood tolerant, although an excess amount of flooding can cause damages to them. Prolonged flooding can lead to anaerobic conditions in the soil, leading to reduced root respiration and eventually fermentation (Pereira and Kozlowski 1977, Levan and Riha 1986, Pezeshki and Chambers 1986, Islam and Macdonald 2004). Fermentation can lead to root tissue dieback due to its inefficient use of carbohydrates (Lambers et al. 2008). However, it remains unclear whether increased carbon dioxide, which has been shown to increase root respiration through increased carbohydrate availability (Nie et al. 2013), can reduce the negative impacts of prolonged flooding on plant morphology and physiology. This thesis has investigated the effects of doubled

atmospheric CO_2 on the physiological and morphological and biomass data responses of *L. laricinia* and *P. mariana* seedlings to a 28-day flooding treatment.

Chapter 2: The physiological responses to flooding under elevated CO2 in *Picea mariana* and *Larix laricina* seedlings

Introduction

The increase in the atmospheric CO_2 concentration has been accelerating due to human activities over the past century (Graven et al. 2013). This increased atmospheric CO_2 has caused an increase in global surface temperatures (IPCC 2007). Changes to precipitation regimes are also expected, with many areas being at an increased risk for flash floods in the summer and more prolonged flooding in the spring and winter (Kreuzwieser and Rennenberg 2014). These changes will likely lead to a rise in the frequency of hydrological events such as flooding in high risk areas (Huntington 2006, Kreuzwieser and Rennenberg 2014).

While increasing atmospheric CO_2 concentration has been detrimental to the abiotic environment, many studies have demonstrated increased photosynthetic capacity (Bazzaz et al. 1993, Drake et al. 1997, Curtis and Wang 1998, Anderson-Teixeira et al. 2013) and photosynthetic water-use efficiency (Keenan et al. 2013) leading to increased plant productivity and biomass accumulation under increased CO_2. Trees are C3 plants and as a result have a high capacity to benefit from increases in carbon dioxide. However, deficiencies in other resources such as nutrients may limit the scope of such beneficial effects (Lambers et al. 2008). In addition to increasing photosynthetic capabilities, increased CO_2 concentration has also been shown to increase root respiration as more carbohydrates are available to be metabolized by the belowground structures (Nie et al. 2013).

When plants are flooded, their roots enter a hypoxic or anoxic environment. The lack of oxygen decreases regular aerobic root respiration, carbon assimilation, and rooting depth in

young conifer trees (Islam and Macdonald 2004). The decrease in oxygen availability leads roots to switch from the normal aerobic respiration pathway to anaerobic fermentation (Kozlowski 1984). Fermentation produces only 1/18 of the energy in the form of ATP per unit of carbohydrate that the aerobic respiration does and thus represents a very inefficient way of energy production and carbohydrate utilization by the roots (Lambers et al. 2008). This inefficiency can lead to declines in many physiological processes within the roots, including water and nutrient uptake (Lambers et al. 2008). Since roots are responsible for the uptake of nutrients and water for the plant to maintain photosynthetic activities, the degradation of roots also leads to a degradation in aboveground processes and a subsequent decline in aboveground productivity (Pereira and Kozlowski 1977, Levan and Riha 1986, Pezeshki and Chambers 1986, Islam and Macdonald 2004). Prolonged hypoxic or anoxic conditions can lead root mortality (Lambers et al. 2008). CO_2 elevations can increase photosynthetic carbon assimilation (Bazzaz et al. 1993, Drake et al. 1997, Curtis and Wang 1998, Anderson-Teixeira et al. 2013) and water-use efficiency, particularly in C3 plants (Keenan et al. 2013). Increases in photosynthetic carbohydrate production can increase the supply of carbohydrates to the root system (Nie et al. 2013) and thus can potentially compensate for its inefficient utilization in the roots under flooded conditions and subsequently the resistance of plants to flooding.

However, to our knowledge, no study has yet examined the effects of CO_2 elevations on the physiological responses of trees to flooding.

P. mariana and *L. laricinia* are found throughout the boreal forest of Canada (Uchytil 1991, Ontario Ministry of Natural Resources 2013a). Both species are also found in a wide variety of topographies, soil types, and soil moisture regimes. *P. mariana* in particular, is the most common commercial tree species in Ontario, making up approximately 37% of the

growing stock in the province (Ontario Ministry of Natural Resources 2013b). *L. laricinia*, while not as popular as *P. mariana*, has some commercial value and provides a natural habitat and food source for many endemic animals. Both species are highly prevalent in the boreal forest on peatlands, which make up approximately 12% of Canada's landmass (Islam and Macdonald 2004, Yang and Yeh 2007). Peatlands are characterized by having a high soil moisture content and poor aeration (Islam and Macdonald 2004). Periodic flooding is a common occurrence in peatlands. Both *P. mariana* and *L. laricinia* have adapted to the wetland site conditions and developed some degree of flood resistance (Islam et al. 2003, Islam and Macdonald 2004). However, it is unknown whether the elevations in atmospheric CO_2 concentration will further enhance their flood resistance and whether the two species would respond differently. There are morphological and physiological differences between the two species, for instance, *L. laricinia* has the capacity to develop adventitious roots when flooded whereas *P. mariana* does not (Calvo-Polanco et al. 2012).

In this study we exposed 2-year-old seedlings of the above two species to a 28-day flooding treatment under the ambient and doubled CO_2 concentrations and investigated the effects of the flooding on a suite of ecophysiological traits and the process of their subsequent recovery. We have tested the hypothesis that the doubling of atmospheric CO_2 concentration would reduce the magnitude of negative impact of flooding on the ecophysiological traits of black spruce and tamarack. Since there are physiological and morphological differences between the two species, e.g., we also examined whether the CO_2 elevation would change the relative flood resistance of the two species. We attempted to unveil the ecophysiological mechanisms of the responses and acclimation of the two species to flooding under different CO_2 concentrations.

Materials and Methods

Seeds

Black spruce seed cones were gathered February 21, 2014 from three felled black spruce trees on a wetland stand in the Lakehead University Jack Haggerty Forest located approximately 37km north of Thunder Bay, ON (48°29′2″ N, 89°29′23″ W). We obtained Tamarack seeds from seed zone 19 (North of Lake Nipigon, Ontario) from the Ontario Tree Seed Centre in Angus, Ontario.

The black spruce seeds were extracted from the cones using the methods described in The Seeds of Woody Plants in the United States (Agriculture Hand Book No. 450 1974). Once the cones were brought back to the greenhouse, they were rinsed and left to soak in cold water for 4 hours. The cones were drained and then left to dry on flat metal trays for 20 hours. Cones were then dried for a total of 15 hours: the drying oven was slowly heated to 54.44ºC (130ºF) over 4 hours and then maintained at that temperature for an additional 11 hours. The cones were left to cool to the room temperature in the oven. After reaching room temperature they were removed from the oven and shaken to extract the seeds.

Seedlings

Black spruce and tamarack seeds were soaked in cold water for 24 hours, after which they were drained and placed in containers covered with moist paper towel. The seeds were then placed in a refrigerator at 4ºC for three weeks to stratify. The seeds were sown in Styrofoam blocks (Beaver Plastics Limited, Acheson, Alberta) with 130 cells of 60ml in volume, with 3-4 seeds per cell. The cells were pre-filled with a mixture of peat moss and vermiculite (3:2 v:v) as the growing medium, and seeding occurred on March 16, 2014. To promote germination and early growth, the Styrofoam blocks were placed in polyethylene misting tents for 34 days. The

environmental conditions of the greenhouse were controlled using an Argus system (Argus Control Systems Ltd. Surrey, British Columbia). The day time temperature was set to 23 ^{o}C and the night time temperature was set to 17 ^{o}C, during the establishment phase. The photoperiod was maintained at 16 hours with an average light intensity of 400 µmol m^{-2} s^{-1}. High pressure sodium lamps were used to extend the natural photoperiod to 16 hours and supplement natural light when it dropped below 400 µmol m^{-2} s^{-1} on average. The seedling containers were misted daily during the germination phase. Plants were determined to need watering when they the volumetric water content of the growing medium reached about 35% as determined using a Delta-T ML2x probe and HH2 moisture meter (Delta-T Devices, Cambridge, UK). The germination of both species was completed by April 7, 2014. The seedlings were moved out of the misting tent on April 25th, 2014.

Once the germination phase was completed the seedlings were watered twice a week with a fertilizer solution of seedling starter (11-41-8: 100.1mg/L N, 373.1 mg/L P and 72.8 mg/L K) for the first two weeks. The fertilizer solution was then changed to the forestry seedling standard fertilizer 20-8-20 (N-P-K). The seedlings were watered as needed; trees were determined to require watering when the volumetric water content of the growing medium reached about 35% as determined using a Delta-T ML2x probe and HH2 moisture meter (Delta-T Devices, Cambridge, UK). The concentration of the forestry standard fertilizer was slowly increased from 50mg/L N, 20mg/L P and 50mg/L K to 200mg/L N, 80mg/L P and 200mg/L K over a period of two months (Forestry Notes, 2009). The average light intensity was 400µmol m^{-2} s^{-1}, while maintaining the 16 hour photoperiod. The day and night time temperatures were set at 22ºC and 17ºC, respectively. The relative humidity in the greenhouse was maintained between 60-70% using an under-bench misting system and monitored by the Argus system.

Cold hardening was initiated on September 22, 2014 when the seedlings reached an average height of 15 cm and was completed December 6, 2014. The trees were hardened using the techniques described in the Container Tree Nursery Manual, volume six (Landis, 1999). The day and night temperature was decreased by 3 ^{o}C/week until the day and night temperatures reached 7 and 4^{o}C respectively, over a five week period, ending on October 27, 2014 (Landis, 1999). The temperatures were then maintained at 7 and 4 ^{o}C, during daytime and nighttime respectively, until November 25, 2014. During those five weeks the photoperiod was also decreased to 8 hours. The shortening of photoperiod was synchronized with the decrease of temperature at a rate of two hours every eight days. A black out curtain was used to ensure that seedlings were in total darkness during the specified dark period. The photoperiod was maintained at eight hours from October 27, 2014 until December 6, 2014. At the start of the hardening phase the fertilizing solution was switched to a conifer seedling finisher fertilizer at a rate of 50mg/L N, 312.5mg/L P and 437.5mg/L K. The frequency of watering was decreased from twice a week to once a week to aid the cold hardening process. The seedlings were determined to be hardened once the roots turned from white to golden brown in colour, the tamarack needles turned yellow or fell off, and buds set on both species. To determine if the root colour had changed two seedlings were pulled from each tray and the roots were washed for the inspection; the washed seedlings were then discarded. Once the seedlings had hardened they were removed from the containers, wrapped in plastic wrap placed in plastic lined boxes in cold storage at -3 ^{o}C on December 6, 2014.

Experimental design

The experiment was conducted in the research greenhouses at Lakehead University in Thunder Bay. The seedlings were moved out of cold storage on January 20, 2015. They were

laid out on slotted trays in a dark room at 17 °C for 24 hours to defrost. The seedlings were then planted in 5×5×6 inch pots with a mixture of peat moss and vermiculite (3:2 v:v). The seedlings were randomly placed on one of the four benches in each of the four greenhouses. Each seedling was randomly assigned a bench placement through a random draw.

The greenhouse conditions from January 20 to January 30, 2015 were ambient light with a 10°C day and a 7°C night temperature. The CO_2 concentration in two of the four greenhouses was set at 400 ppm which represents the current ambient atmospheric CO_2 concentration (National Oceanic and Atmospheric Administration Earth System Research Laboratory, Mauna Loa, Hawaii, United States of America http://www.esrl.noaa.gov/gmd/ccgg/trends/index.html) while the other two greenhouses were set to an 800 ppm CO_2 concentration. The day and night temperatures were increased by 1.5°C per week for 7 weeks. No supplement light was used during the first two weeks of the experiment. Subsequently the natural light was supplemented using high pressure sodium lamps (400 µmol m^{-2} s^{-1} PAR on average) to supplement a photoperiod of 8 hours at 400 µmol m^{-2} s^{-1} PAR. From February 3 until March 9 the supplement light was gradually increased to 16 hours (at a rate of 2 hours a week). The seedling pots were placed in clear plastic bins (10 seedlings per bin), which were used later for flooding treatment. To avoid any confounding effects by the bins, control seedlings were also placed in identical bins in the same manner. The temperature and light conditions were slowly increased over this period to emulate the average conditions during the spring to summer transition until the seedlings flushed their buds, signifying that they were actively growing (Islam and MacDonald, 2003). During the seven weeks leading up to the start of the flooding treatment the seedlings were watered when the volumetric water content of the growing medium reached about 35% as described previously. Starting two weeks after the start of the experiment, the seedlings were

fertilized with a standard forestry fertilizer solution of 50mg/L N, 20mg/L P, and 50mg/L K once a week for the remainder of the experiment.

The flooding treatment lasted a total of 28 days. Flooding was simulated by filling the plastic bin with water up to the seedlings' root collar positon. The control group was watered when soil moisture dropped between 5% as described previously. The control bins had six 0.5 cm holes drilled in the bottom to ensure that any excess water was able to drip out of the bin. The flooded bins were drained completely on May 1, 2015 and left to recover until June 5, 2015. The experiment was a split plot design, with the split at the at the greenhouse level. CO_2 treatment was a whole plot factor and flooding and species were sub-plot factors. A total of four greenhouses were used creating two replicates of the whole plot: making 2 greenhouses per CO_2 treatment. There were a total of eight sub-plot replicates within each whole plot replicate. There were a total of four benches in each of the four greenhouses (totalling 16 benches). There were two bins (flooded and control) placed on each of the benches and 5 seedlings of each species in each bin. A total of 160 seedlings of each species were used for the entire experiment. The location of each seedling within the bin was randomized. The bin to be flooded was determined by coin flip.

Measurements

To determine the physiological changes in the above and below ground structures of the seedlings photosynthetic rate, stomatal conductance, root respiration and foliar chlorophyll fluorescence. Photosynthesis, stomatal conductance and root respiration were measured using a PP-Systems CIRAS-3 open gas exchange system and a Parkinson conifer leaf chamber with automatic environment control (PP System Inc., Amesbury, MA, USA) and chlorophyll

fluorescence was measured using a FMS-2 portable pulse-modulated fluorometer (Hansatech Instruments Ltd. Norfolk, UK).

The first gas exchange measurements were taken 17 days after the start of flooding. The seedlings in the ambient CO_2 were measured at the ambient CO_2 concentration (400 ppm) while those grown in the doubled CO_2 (800 ppm) were measured at 800. The light level for the measured was set to match the average growing conditions (400µmol m^{-2} s^{-1}). Readings were recorded when they stabilized. Five readings were taken for each sample and averaged to get the final number used in the data analysis. The second round of measurements of photosynthesis and stomatal conductance were taken 6 days after flooding had stopped. The measurements were taken on 64 seedlings (4 seedlings/ species/ flood treatment/ greenhouse) and the foliage that was measured was marked with black permanent marker for the repeat of the measurement in June and determining the leaf area. The foliar gas exchange was repeated 35 days following the termination of the flood treatment on the same seedling and same foliage as the May measurement. The environmental conditions and PPS settings were consistent in all the gas exchange measures and described previously. Following the measurements, the sample foliage was cut and scanned using Regent WinSEEDLE (Regent Instruments Inc., Quebec, Canada) to determine the leaf area. Because of the destructive nature of the measurement and the limited number of seedlings, root respiration was taken only twice: 17 days after flooding and after a 35-day recovery period. The roots were extracted from the container, washed and water on the surface of all roots was removed using paper towel before being placed in the chamber for measurement as described previously for foliar gas exchange measurements. However, the root respiration was measured in darkness. A total of 64 seedlings were measured on April 21st, 2015: 4 seedlings/ species/ flood treatment/ greenhouse. Since measuring root respiration was

destructive these seedlings were not returned to the experiment following the measurement. The roots enclosed in the measurement chamber were cut from the rest of the plant following the measurements and placed in labeled bags and stored in a freezer. They subsequently were scanned using Regent WinRHIZO (Regent Instruments Inc., Quebec, Canada) to determine the root area and root length

To determine the maximum quantum yield of Photosystem II, chlorophyll fluorescence was measured during and following the flooding treatment in increments of four days during flooding and weekly during recovery, starting six days after the start of the flooding (April 10th, 2015) and ending 35 days after the termination of the treatment (June 5th, 2015, measured 10 times in total). The measurement was taken on four randomly selected seedlings from each species by treatment combination after the foliage was dark adapted for at least 60 minutes. Since *L. laricina* only has current year foliage while *P. mariana* has both current year and previous year foliage, the measurements were taken on the current year foliage in both species to make them more comparable. Three readings were recorded on each sample and the average was used in the subsequent data analysis.

Data Analysis

The data was analyzed using a split-plot ANOVA, with CO_2 treatment at the whole plot level, and flooding treatment and species at the subplot level. The assumptions of normality and homogeneity were assessed using the Shapiro tests and examining the residual plots respectively. Since both assumptions were met for all the variables, no data transformation was necessary. The full model used was:

$$Y_{ijkl} = C_i + \omega_{(i)j} + \delta_{(ij)} + F_k + CF_{jk} + \omega F_{(i)jk} + S_l + CS_{il} + \omega S_{(i)l} + CFS_{ikl} + \omega FS_{(i)jkl} + \varepsilon_{(ijkl)m}$$

where: i= 2 levels of CO_2, j= whole plot or greenhouses, k= 2 Flooding, l= 2 species, m= 4 subplot replicate, ω= whole plot error, δ= restriction due to randomization, S= Species, F= Flood Treatment, C= CO_2 level (Table 2-1.). When ANOVA showed a significant interaction, a Tukey's post-hoc test was used to compare the means. All the analyses were conducted using the "R" statistics software ("R", Geneva Switzerland).

Table 2-1. EMS table of Statistical Model used for the analysis of data.

	2 F i	2 R j	2 F k	2 F l		
						df
C_i	0	2	2	2	$\sigma^2 + 4\,\sigma^2\omega + 8\Phi C$	1
$\omega_{(i)j}$	1	1	2	2	$\sigma^2 + 4\,\sigma^2\omega$	2
$\delta_{(ij)}$	1	1	2	2	$\sigma^2 + 4\,\sigma^2\delta_{(ij)}$	0
F_k	2	2	0	2	$\sigma^2 + 2\,\sigma^2\omega F + 8\Phi F$	1
CF_{ik}	0	2	0	2	$\sigma^2 + 2\,\sigma^2\omega F + 4\Phi CF$	1
$\omega F_{(i)jk}$	1	1	0	2	$\sigma^2 + 2\,\sigma^2\omega F$	2
S_l	2	2	2	0	$\sigma^2 + 2\,\sigma^2\omega S + 8\Phi S$	1
CS_{il}	0	2	2	0	$\sigma^2 + 2\,\sigma^2\omega S + 4\Phi CS$	1
$\omega S_{(i)jl}$	1	1	2	0	$\sigma^2 + 2\,\sigma^2\omega S$	2
FS_{kl}	2	2	0	0	$\sigma^2 + \sigma^2\omega FS + 4\Phi FS$	1
CFS_{ikl}	0	2	0	0	$\sigma^2 + \sigma^2\omega FS + 2\Phi CFS$	1
$\omega FS_{(i)jkl}$	1	1	0	0	$\sigma^2 + \sigma^2\omega FS$	2
$\varepsilon_{(ijkl)}$	1	1	1	1	σ^2	48
						=63 df

Results

Photosynthetic rate was not significantly affected by the CO_2 or flooding treatment for either species after 17 days of flooding or after 35 days following the termination of the flooding treatment. However, the photosynthetic rate was significantly lower in the flooded (0.06 µmol

m^{-2} s^{-1}) than the control (0.18 μmol m^{-2} s^{-1}) 6 days after the termination of the flooding treatment (Table 3-2). Although the CO_2 elevation significantly increased photosynthesis in both species, the magnitude of increase was much greater in *L. laricina* than in *P. mariana* (Figure 3-1, Figure 3-2 A and B, Table 3-2).

Table 2-2. The probability of effects of Doubled CO_2, Flooding and Species on photosynthesis (*A*) and stomatal conductance (g_s), where probabilities are in bold (</= 0.05).

Terms	**17 days of flooding**		**6 days of recovery**		**35 days of recovery**	
	A	**g_s**	**A**	**g_s**	**A**	**g_s**
CO_2	0.846	**0.029**	**0.010**	**0.025**	0.885	0.649
Flooding	0.200	0.174	**0.027**	**0.048**	0.105	0.107
CO_2 X Flooding	0.393	0.845	0.185	0.853	0.787	0.470
Species	0.798	0.543	**0.009**	**0.025**	0.889	0.275
CO_2 X Species	0.838	0.523	**0.028**	0.440	0.228	0.164
Flooding X Species	0.397	0.775	0.087	0.266	0.160	0.841
CO_2 X Flooding X Species	0.452	0.830	0.151	0.845	0.422	0.693

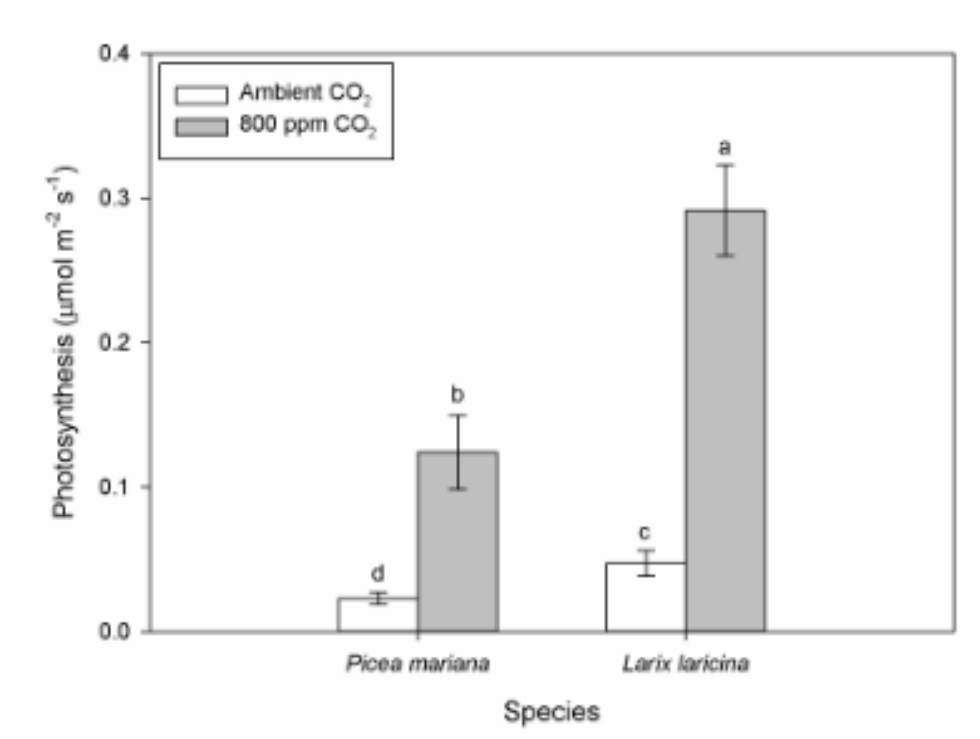

Figure 2-1. The interactive effects of CO_2 concentration and species on photosynthesis 6 days following the termination of flood treatment.

The doubled CO_2 significantly reduced stomatal conductance 17 days into flooding (Ambient: 6.55 mmol m^{-2} s^{-1}, Doubled: 1.12 mmol m^{-2} s^{-1}) but significantly increased it 6 days after flooding had ceased (Ambient: 0.75 mmol m^{-2} s^{-1}, Doubled: 2.39 mmol m^{-2} s^{-1}), while there was no significant effect 35 days after the termination of the flood treatment (Table 3-2). Flooded seedlings had significantly lower stomatal conductance 6 days after flooding had ceased (Flood: 0.83 mmol m^{-2} s^{-1}, Control: 2.31 mmol m^{-2} s^{-1}) (Table 3-2). Flooding had no significant effects on stomatal conductance in the other two measurements. *L. laricina* had a higher stomatal conductance (2.38) 6 days after flooding had ceased than *P. mariana* (0.76) (Figure 3-2 C and D, Table 3-2).

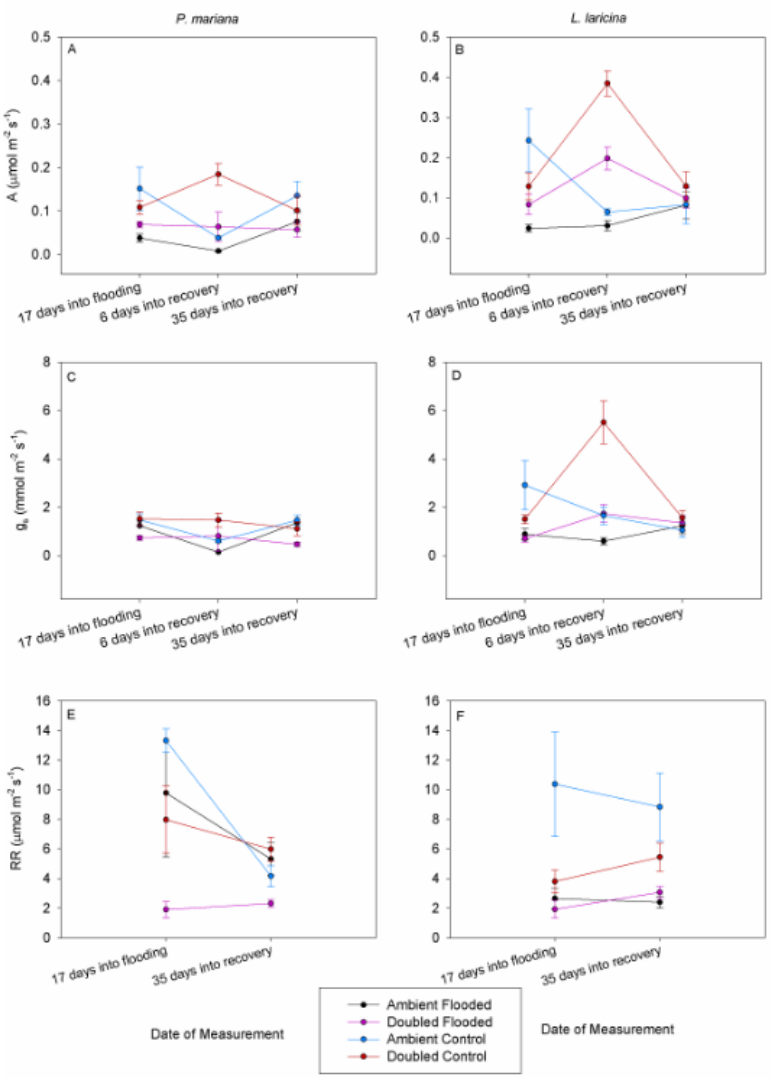

Figure 2-2. The effects of Doubled CO_2 and flooding on A) the photosynthesis (*A*) of *P. mariana* and B) the photosynthesis of *L. laricina* where A represents photosynthesis and days represents time of measurement. The effects of Doubled CO_2 and flooding on C) the stomatal conductance (g_s) of *P. mariana* and D) the stomatal conductance of *L. laricina*. The effects of Doubled CO_2 and flooding on E) the root respiration (RR) of *P. mariana* and F) the root respiration of *L. laricina*.

Root respiration was significantly reduced in flooded seedlings 17 days into the flooding period (Flood: 7.43 μmol m^{-2} s^{-1}, Control: 8.87 μmol m^{-2} s^{-1}) and at the end of the experiment 35 days after flooding had ceased (Flood: 3.28 μmol m^{-2} s^{-1}, Control: 5.11 μmol m^{-2} s^{-1}) (Figure 3-2 E and F, Table 3-3). Root respiration was significantly increased in doubled CO_2 35 days after flooding had ceased (Ambient: 4.18 μmol m^{-2} s^{-1}, Doubled: 4.20 μmol m^{-2} s^{-1}) (Table 3-3).

Table 2-3. The probability of effects of Doubled CO_2, Flooding and Species on Root Respiration (RR) after 17 days of flooding and 35 days after the termination of the 28 day flooding treatment. Where significant probabilities are in bold (</= 0.05)

Terms	RR 17 days of Flooding	RR 35 of Recovery
CO_2	0.157	**0.003**
Flooding	**0.003**	**0.007**
CO_2 X Flooding	**0.060**	0.100
Species	0.127	0.956
CO_2 X Species	0.495	0.643
Flooding X Species	0.789	0.273
CO_2 X Flooding X Species	0.394	0.110

After 22 days of flooding, the maximum quantum yield of Photosystem II, Fv/Fm, was significantly reduced and this significant effect persisted until 35 days after the termination of the flooding treatment when the difference in Fv/Fm became insignificant (Figure 3-5, Table 4). The

effects of other factors or interactions between factors were generally not significant throughout the experiments with the exceptional of a couple of measurements (Table 4, Figures 3-3 and 4).

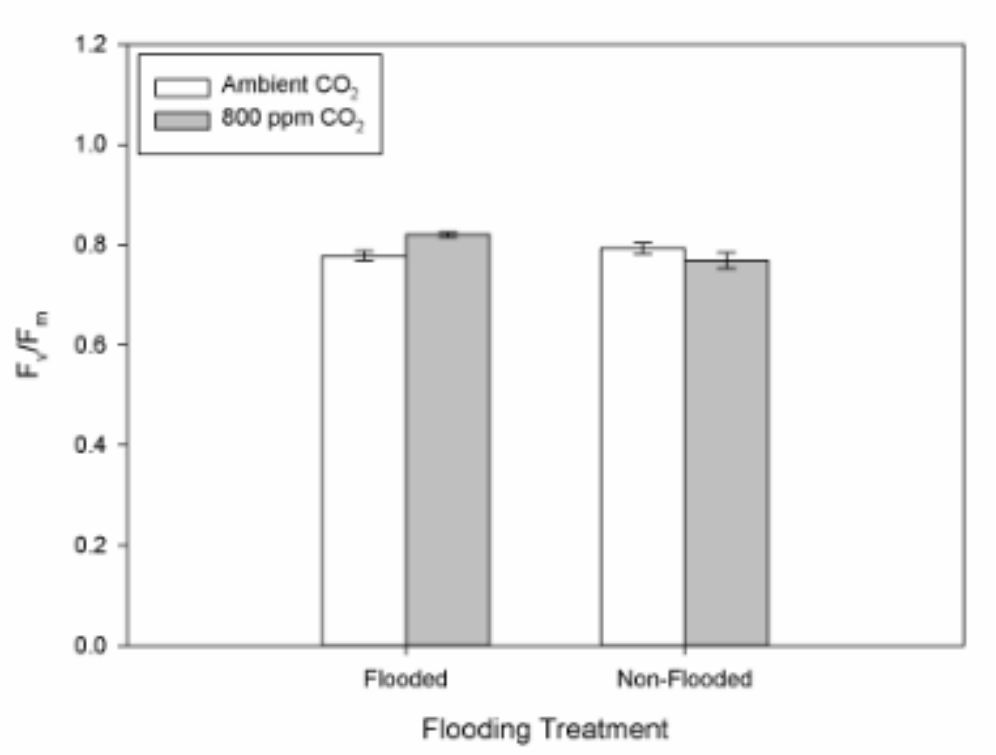

Figure 2-3. The interaction between CO_2 concentration and flooding on the maximum quantum yield of Photosystem II (Fv/Fm) on April 10th, 2015 (6 days after flooding).

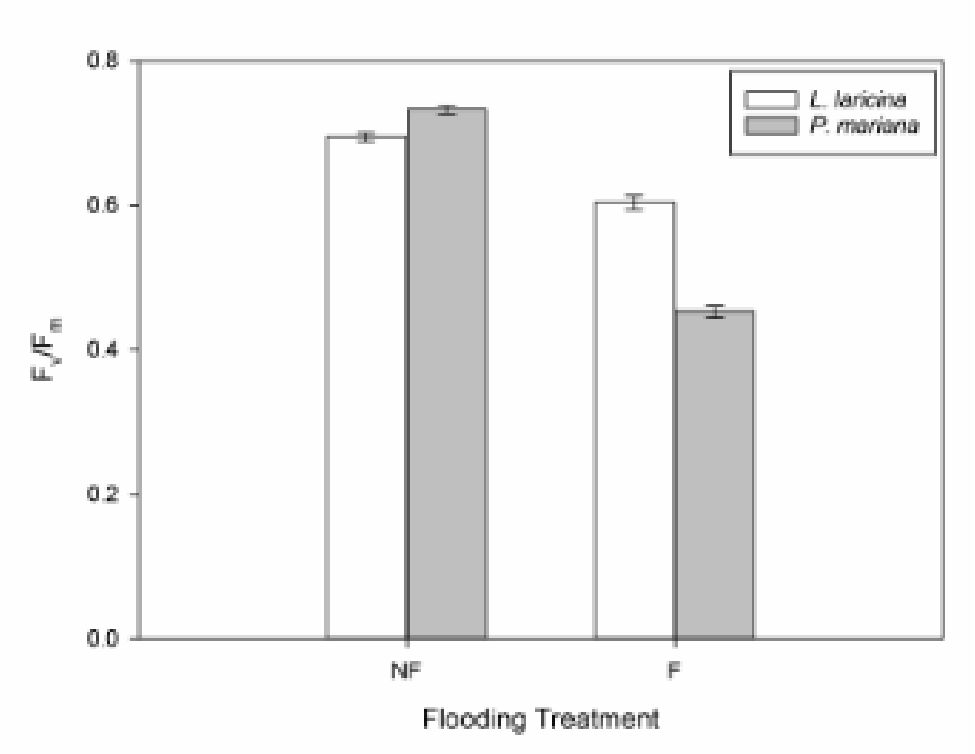

Figure 2-4. The interaction between flooding and species on the maximum quantum yield of Photosystem II (Fv/Fm) on April 26th 2015: after 22 days after flooding.

Table 2-4.The effects of CO_2, Flooding and Species on the maximum quantum yield of Photosystem II (Fv/Fm), from 6 days after flooding until 35 days after recovery. Significant probabilities are bolded (</= 0.05).

Terms	**04/10/15**	**04/14/15**	**04/22/15**	**04/26/15**	**04/30/15**	**05/06/15**	**05/10/15**	**05/14/15**	**05/18/15**	**06/05/15**
CO_2	0.143	0.501	0.955	0.595	0.392	0.240	0.007	0.110	0.042	0.874
Flooding	0.163	0.254	0.311	0.016	0.005	0.013	0.007	0.008	0.002	0.006
CO_2 X Flooding	0.0141	0.087	0.679	0.517	0.193	0.818	0.127	0.104	0.073	0.806
Species	0.867	0.878	0.997	0.319	0.133	0.337	0.192	0.090	0.108	0.076
CO_2 X Species	0.122	0.181	0.464	0.837	0.623	0.313	0.072	0.215	0.102	0.311
Flooding X Species	0.481	0.368	0.376	0.049	0.238	0.168	0.266	0.060	0.128	0.098
CO_2 X Flooding X Species	0.904	0.815	0.163	0.210	0.947	0.355	0.119	0.095	0.118	0.366

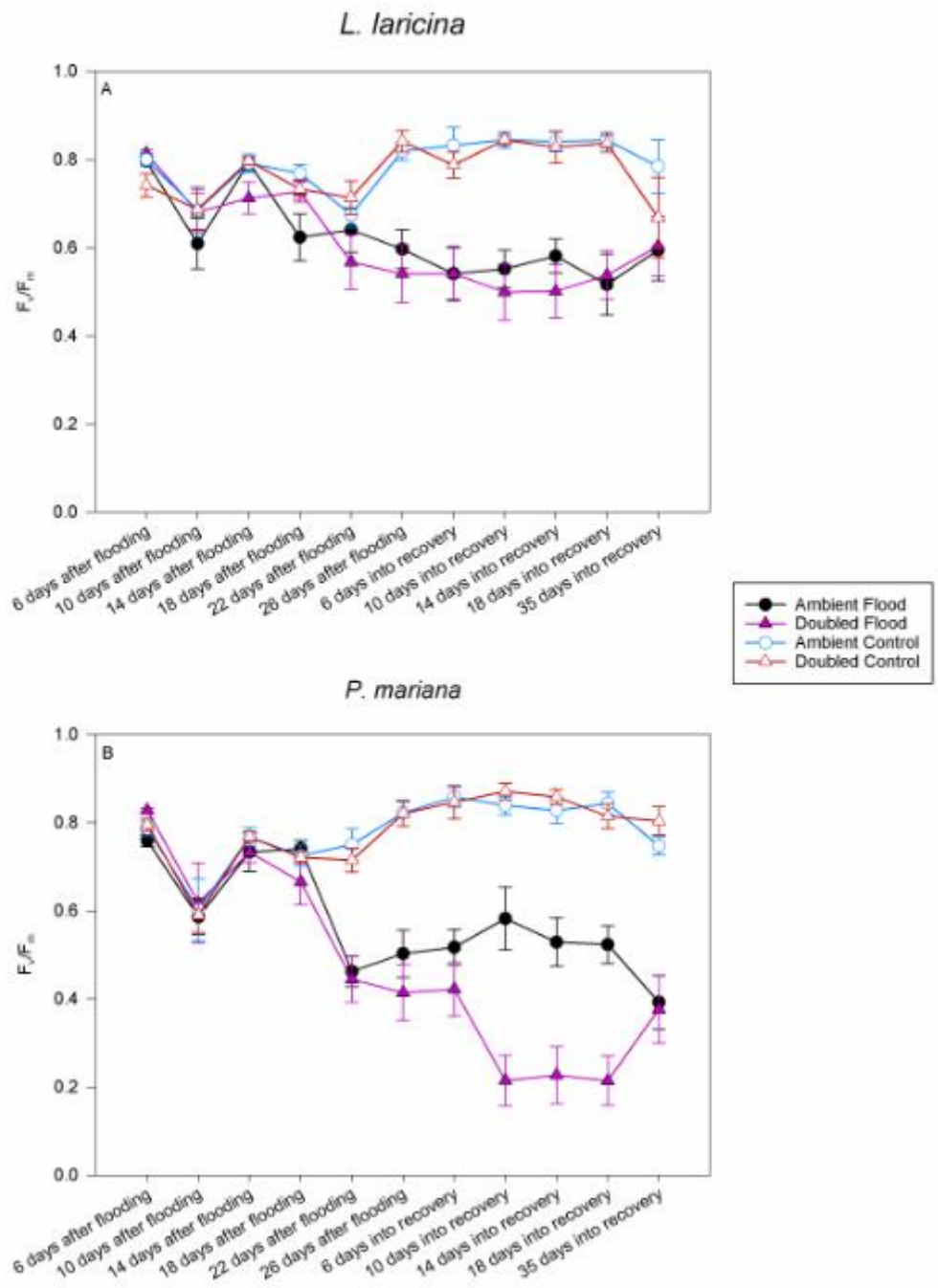

Figure 2-5. The effects of Doubled CO_2 and flooding on A) the chlorophyll fluorescence of *P. mariana* and B) *L. laricina*.

Discussion

The results of this study do not support the hypothesis that CO_2 elevations would reduce the degree of damage of and assist the recovery from flooding. The basic assumption for the hypothesis is that CO_2 elevations will increase photosynthetic carbohydrate production that will in turn compensate the inefficient use of carbohydrates by roots under flooded conditions. However, the CO_2 elevation in this study did not lead to sustained increases in photosynthesis. This may be attributable to photosynthetic down regulation. CO_2 elevations generally lead to increases in photosynthesis initially. However, longer term exposure can lead to a down regulation of photosynthetic capacity and a reduction in biomass allocation to leaves with a concurrent increase in root biomass ((Islam and Macdonald 2004)). Furthermore, CO_2 elevations lead to increased CO_2 dissolution in water and thus could have lowered the pH in the rhizosphere in the flooded treatment. A Lower pH could have exacerbated flooding induced injuries to roots (Lambers et al. 208). This negative effect could offset, at least partially, the possible positive effect of the CO_2 elevation on the physiological response to flooding.

We have found that Photosystem II was more sensitive to flooding than photosynthetic gas exchange in black spruce and tamarack. There was no consistent decline in photosynthetic CO_2 assimilation or stomatal conductance during or after the flooding treatment. However, the chlorophyll fluorescence measurement, Fv/Fm, was consistently lower in seedlings of both species in the flooding treatment, starting from 22^{nd} day of flooding until 35 days after the termination of the flooding treatment. Fv/Fm is indicative of the quantum efficiency of electron transport in Photosystem II, which is an essential part of the photosynthetic apparatus where photosynthetic water splitting and oxygen production occur (Lambers et al. 2008). Photosystem II is generally more sensitive to environmental stresses than the dark reactions of photosynthesis

(Lambers et al. 2008). The fact that there was no significant decrease in Fv/Fm in the first weeks of flooding suggests that the species had the capacity to tolerate short term flooding without suffering physiological injuries.

Flooding reduced photosynthesis and stomatal conductance in black spruce and tamarack only in the measurements made six days after flooding had ceased. While the decreases are consistent with the findings of other studies (Pereira and Kozlowski 1977, Levan and Riha 1986, Pezeshki and Chambers 1986, Islam and Macdonald 2004), the lack of significant flooding effects in the other measurements, particularly those made after 17 days of flooding, is contradictory. However, the chlorophyll fluorescence data indicate that Photosystem II activities in the flooded seedlings remained depressed during and after the flood treatment and only recovered after an extended period of time following the termination of the flood treatment. The lack of consistent significant flooding effects on photosynthesis and stomatal conductance may have been due to the small sample size for the gas exchange measurements and the conservative nature of the split plot design of the experiment. Additionally the responses in photosynthesis and stomatal would probably have been smaller, if existed, than that of chlorophyll fluorescence because gas exchanges tend to be less sensitive stresses than chlorophyll fluorescence.

The flooding effect on root respiration was more obvious than that on foliar gas exchange. Flooding decreased root respiration both during the flood treatment and 35 days after the termination of the treatment. This is in a general agreement with the literature (Pereira and Kozlowski 1977, Levan and Riha 1986, Pezeshki and Chambers 1986, Islam and Macdonald 2004). This suggests that while aboveground organs may be able to tolerate a 28 day episode of flooding and their functions eventually recover after the termination of the treatment, the injuries to roots and root function were more severe and may take longer to recover. However, the

physiological mechanisms responsible for the reduction in root respiration may be different between the two measurements. Flooding leads to a hypoxic condition will inhibit the normal cytochrome respiration in roots and induce fermentation, leading to a lower measurement of CO_2 release (Lambers et al. 2008). The anoxic and hypoxic conditions often also lead to root mortality, particularly fine roots. Fine roots have greater physiological activities and thus higher rate of respiration than courser roots. The loss of fine roots was probably the primary cause for the lower rates of root respiration measured 35 days following the cessation of flooding. A significant reduction in root mass and root mass ratio were indeed observed in this study.

The two species did not show any significant differences in their responses to flooding either under the ambient or the doubled CO_2 concentration. Both species naturally grow on lowland sites where periodic flooding occurs throughout the year ((Islam and Macdonald 2004, Yang and Yeh 2007, Ballantyne et al. 2014). It is, therefore, not surprising that the two species has similar responses to the flooding treatment. However, the mechanisms of the responses might have differed, for example, *L. laricina* developed adventitious roots in the flooding treatments while *P. mariana* did not. The development of adventitious roots in *L. laricina* is also observed in other studies (Calvo-Polanco et al. 2012). The adventitious roots had likely contributed to the higher stomatal conductance and presumably higher transpiration in *L. laricina* than *P. mariana* during the initial recovery period. The Fv/Fm measurements during the same time period were greater in the flooded *l. laricina* than in the flooded *P. mariana*, but the difference was statistically not significant. The data suggests that *L. laricina* probably experienced relatively less stress from the flooding treatment, at least partly due to their ability to develop adventitious roots to mitigate the effect of flooding stress.

As mentioned previously, the limited number of significant interactions was likely due in part to the statistical model used. Split-plot ANOVAs tend to be conservative as they split errors into smaller groups than a typical ANVOA with smaller degrees of freedom. Though they are conservative in nature, a split-plot ANOVA was necessary for this experiment due to the experimental design. In order to have the proper numbers of seedlings for replication we used four greenhouses, which left us with two degrees of freedom in the whole plot error – significantly less than the 48 degrees of freedom left in the innermost residuals.

Chapter 3: The responses of to flooding under elevated CO2: Growth, mortality and morphological characteristics of *Larix laricina* and *Picea mariana*

Introduction

Atmospheric CO_2 concentration has been increasing in the past century due to human activities (Graven et al. 2013). This increased atmospheric CO_2 is considered to be a key factor for the increases in global temperature (IPCC 2007) and increases in the frequency of hydrological events such as flooding (Huntington 2006, Kreuzwieser and Rennenberg 2014). Elevated CO_2 is expected to increase both the length and diameter of roots, and stimulate coarse, fine, and total root biomass (Nie et al. 2013). Elevated CO_2 also changes the distribution of roots within the vertical soil profile and root dynamics, such as a deeper rooting distribution as well as an increased root turnover rate (Nie et al. 2013). Elevated CO_2 may also increase photosynthetic carbon assimilation, aboveground productivity and total gross primary production (Anderson-Teixeira et al. 2013). While these increases have been smaller in Free-Air CO_2 Enrichment experiments than expected, increases have still been observed, likely related to CO_2 elevation induced increases in water-use efficiency (Leakey et al. 2009).

The high water tables in flooded soils reduce the amount of oxygen available to tree roots, causing the change in root respiration pathway from aerobic respiration to the more inefficient anaerobic fermentation (Lambers et al. 2008). Oxidative phosphorylation does not function in flooded condition, causing glycolysis to stop. In order to overcome this limitation plants switch to alcoholic fermentation (Taiz and Zeiger, 2010). In alcoholic fermentation two enzymes (pyruvate decarboxylase and alcohol dehydrogenase) act on pyruvate which eventually produces ethanol and CO_2: oxidizing NADH as a result (Taiz and Zeiger, 2010). Fermentation decreases the efficiency of ATP production by increasing the amount of carbohydrates required to produce the same amount of ATP (Taiz and Zeiger, 2010). This switch can lead to decreases in carbon assimilation rates and rooting depth (Islam and Macdonald 2004). As roots are responsible for the uptake of nutrients and water (Grant et al. 2012, Nie et al. 2013), loss in roots and reduction in root function cause a reduction in aboveground productivity (Islam and Macdonald 2004). Longer periods of flooding exacerbate these negative effects (Parad et al. 2013).

Despite that increases in atmospheric CO_2 and likely increases in flooding have been well documented, the effects of these two variables in combination on plants have not been studied. Since the major direct effect of flooding is to cause reductions in root physiological functions and eventually root death due to inefficient use of carbohydrate, increased atmospheric CO_2 could help alleviate these negative effects by increasing plant water-use efficiency and photosynthetic carbon assimilation and thus carbohydrate supply to the flooded roots. However, increasing atmospheric CO_2 has been shown to deepen rooting distribution, which may leave roots more susceptible to the effects of flooding.

The Canadian boreal forest has a wide range of topography, with peatlands making up nearly 12% of the total landmass (Yang and Yeh 2007). Two of the dominant tree species in these peatlands are *Picea mariana* (Mill.) and *Larix laricina* (Du Roi) (Uchytil 1991, Ontario Ministry of Natural Resources 2013a). These two species occur in a wide variety of environments from coast to coast, with *P. mariana* making up nearly 37% of the province of Ontario's growing stock (Ontario Ministry of Natural Resources 2013b). Both species experience periodic flooding in peatlands. This periodic flooding can be detrimental to trees if prolonged and intense, but some natural flooding is vital to the functioning of these ecosystems (Benke 2001, Mosepele et al. 2009), as the flooding water carries nutrients to the ecosystem (Junk et al. 1989, Sparks 1995, Tockner et al. 2000). Both species are flood tolerant. *L. laricina* has the ability of producing adventitious roots when flooded to facilitate oxygen transports to the flooded roots (Kreuzwieser and Rennenberg 2014). This provides an interesting comparison as the effects of CO_2 elevation on a species capable of producing adventitious roots could be different than those on a species without that capability.

In this study we flooded the seedlings of the two species for 28 days under the ambient and doubled atmospheric CO_2 concentrations and investigated the morphological responses and subsequent recovery. We tested the following hypothesis: the doubled atmospheric CO_2 would improve seedling flood tolerance and thus reduce the negative effects of the flooding. It is expected that the two species will respond differently to the treatments due to their physiological and morphological differences, so this will also be examined.

Materials and Methods

Seeds

Black spruce seed cones were gathered February 21, 2014 from three felled black spruce trees on a wetland stand in the Lakehead University Jack Haggerty Forest located approximately 37km north of Thunder Bay, ON (48°29′2″ N, 89°29′23″ W). We obtained Tamarack seeds from seed zone 19 (North of Lake Nipigon, Ontario) from the Ontario Tree Seed Centre in Angus, Ontario.

The black spruce seeds were extracted from the cones using the methods described in The Seeds of Woody Plants in the United States (Agriculture Hand Book No. 450 1974). Once the cones were brought back to the greenhouse, they were rinsed and left to soak in cold water for 4 hours. The cones were drained and then left to dry on flat metal trays for 20 hours. Cones were then dried for a total of 15 hours: the drying oven was slowly heated to 54.44ºC (130ºF) over 4 hours and then maintained at that temperature for an additional 11 hours. The cones were left to cool to the room temperature in the oven. After reaching room temperature they were removed from the oven and shaken to extract the seeds.

Seedlings

Black spruce and tamarack seeds were soaked in cold water for 24 hours, after which they were drained and placed in containers covered with moist paper towel. The seeds were then placed in a refrigerator at 4ºC for three weeks to stratify. The seeds were sown in Styrofoam blocks (Beaver Plastics Limited, Acheson, Alberta) with 130 cells of 60ml in volume, with 3-4 seeds per cell. The cells were pre-filled with a mixture of peat moss and vermiculite (3:2 v:v) as the growing medium, and seeding occurred on March 16, 2014. To promote germination and early growth, the Styrofoam blocks were placed in polyethylene misting tents for 34 days. The

environmental conditions of the greenhouse were controlled using an Argus system (Argus Control Systems Ltd. Surrey, British Columbia). The day time temperature was set to 23 ^{o}C and the night time temperature was set to 17 ^{o}C, during the establishment phase. The photoperiod was maintained at 16 hours with an average light intensity of 400 µmol m^{-2} s^{-1}. High pressure sodium lamps were used to extend the natural photoperiod to 16 hours and supplement natural light when it dropped below 400 µmol m^{-2} s^{-1} on average. The seedling containers were misted daily during the germination phase. Plants were determined to need watering when they the volumetric water content of the growing medium reached about 35% as determined using a Delta-T ML2x probe and HH2 moisture meter (Delta-T Devices, Cambridge, UK). The germination of both species was completed by April 7, 2014. The seedlings were moved out of the misting tent on April 25th, 2014.

Once the germination phase was completed the seedlings were watered twice a week with a fertilizer solution of seedling starter (11-41-8: 100.1mg/L N, 373.1 mg/L P and 72.8 mg/L K) for the first two weeks. The fertilizer solution was then changed to the forestry seedling standard fertilizer 20-8-20 (N-P-K). The seedlings were watered as needed; trees were determined to require watering when the volumetric water content of the growing medium reached about 35% as determined using a Delta-T ML2x probe and HH2 moisture meter (Delta-T Devices, Cambridge, UK). The concentration of the forestry standard fertilizer was slowly increased from 50mg/L N, 20mg/L P and 50mg/L K to 200mg/L N, 80mg/L P and 200mg/L K over a period of two months (Forestry Notes, 2009). The average light intensity was 400µmol m^{-2} s^{-1}, while maintaining the 16 hour photoperiod. The day and night time temperatures were set at 22ºC and 17ºC, respectively. The relative humidity in the greenhouse was maintained between 60-70% using an under-bench misting system and monitored by the Argus system.

Cold hardening was initiated on September 22, 2014 when the seedlings reached an average height of 15 cm and was completed December 6, 2014. The trees were hardened using the techniques described in the Container Tree Nursery Manual, volume six (Landis, 1999). The day and night temperature was decreased by 3 ^{o}C/week until the day and night temperatures reached 7 and 4^{o}C respectively, over a five week period, ending on October 27, 2014 (Landis, 1999). The temperatures were then maintained at 7 and 4 ^{o}C, during daytime and nighttime respectively, until November 25, 2014. During those five weeks the photoperiod was also decreased to 8 hours. The shortening of photoperiod was synchronized with the decrease of temperature at a rate of two hours every eight days. A black out curtain was used to ensure that seedlings were in total darkness during the specified dark period. The photoperiod was maintained at eight hours from October 27, 2014 until December 6, 2014. At the start of the hardening phase the fertilizing solution was switched to a conifer seedling finisher fertilizer at a rate of 50mg/L N, 312.5mg/L P and 437.5mg/L K. The frequency of watering was decreased from twice a week to once a week to aid the cold hardening process. The seedlings were determined to be hardened once the roots turned from white to golden brown in colour, the tamarack needles turned yellow or fell off, and buds set on both species. To determine if the root colour had changed two seedlings were pulled from each tray and the roots were washed for the inspection; the washed seedlings were then discarded. Once the seedlings had hardened they were removed from the containers, wrapped in plastic wrap placed in plastic lined boxes in cold storage at -3 ^{o}C on December 6, 2014.

Experimental design

The experiment was conducted in the research greenhouses at Lakehead University in Thunder Bay. The seedlings were moved out of cold storage on January 20, 2015. They were

laid out on slotted trays in a dark room at 17 ^{o}C for 24 hours to defrost. The seedlings were then planted in 5×5×6 inch pots with a mixture of peat moss and vermiculite (3:2 v:v). The seedlings were randomly placed on one of the four benches in each of the four greenhouses. Each seedling was randomly assigned a bench placement through a random draw.

The greenhouse conditions from January 20 to January 30, 2015 were ambient light with a 10^{o}C day and a 7^{o}C night temperature. The CO_2 concentration in two of the four greenhouses was set at 400 ppm which represents the current ambient atmospheric CO_2 concentration (National Oceanic and Atmospheric Administration Earth System Research Laboratory, Mauna Loa, Hawaii, United States of America http://www.esrl.noaa.gov/gmd/ccgg/trends/index.html) while the other two greenhouses were set to an 800 ppm CO_2 concentration. The day and night temperatures were increased by 1.5^{o}C per week for 7 weeks. No supplement light was used during the first two weeks of the experiment. Subsequently the natural light was supplemented using high pressure sodium lamps (400 µmol m^{-2} s^{-1} PAR on average) to supplement a photoperiod of 8 hours at 400 µmol m^{-2} s^{-1} PAR. From February 3 until March 9 the supplement light was gradually increased to 16 hours (at a rate of 2 hours a week). The seedling pots were placed in clear plastic bins (10 seedlings per bin), which were used later for flooding treatment. To avoid any confounding effects by the bins, control seedlings were also placed in identical bins in the same manner. The temperature and light conditions were slowly increased over this period to emulate the average conditions during the spring to summer transition until the seedlings flushed their buds, signifying that they were actively growing (Islam and MacDonald, 2003). During the seven weeks leading up to the start of the flooding treatment the seedlings were watered when the volumetric water content of the growing medium reached about 35% as described previously. Starting two weeks after the start of the experiment, the seedlings were

fertilized with a standard forestry fertilizer solution of 50mg/L N, 20mg/L P, and 50mg/L K once a week for the remainder of the experiment.

The flooding treatment lasted a total of 28 days. A period of 28 days was used for the flooding period due to high rates of mortality seen at the 28 day mark of the experiment. Originally the flood period was going to be 35 days based on previous literature, but the high mortality of *P. mariana* seen at the 28 day mark threatened to reduce the number of viable seedlings below what was needed for measurements. As a result the decision was made to terminate flooding one week ahead of schedule to ensure proper measurements could be taken. Flooding was simulated by filling the plastic bin with water up to the seedlings' root collar positon. The control group was watered when soil moisture dropped between 5% as described previously. The control bins had six 0.5 cm holes drilled in the bottom to ensure that any excess water was able to drip out of the bin. The flooded bins were drained completely on May 1, 2015 and left to recover until June 5, 2015.

The experiment was a split plot design, with the split at the at the greenhouse level. CO_2 treatment was a whole plot factor and flooding and species were sub-plot factors. A total of four greenhouses were used creating two replicates of the whole plot: making 2 greenhouses per CO_2 treatment. There were a total of eight sub-plot replicates within each whole plot replicate. There were a total of four benches in each of the four greenhouses (totalling 16 benches). There were two bins (flooded and control) placed on each of the benches and 5 seedlings of each species in each bin. A total of 160 seedlings of each species were used for the entire experiment. The location of each seedling within the bin was randomized. The bin to be flooded was determined by coin flip.

Measurements

To determine changes in seedling growth and morphology a combination of WinRHIZO and hand taken measurements were used to determine biomass, biomass ratios, relative height growth rate, relative growth rate of root collar diameter, specific leaf area, specific root length and root surface area, and, volume. To determine the relative growth rates of height and root collar diameter, the height and diameter of all seedlings were taken on the first day of flooding and at the end of the experiment. Four seedlings per treatment combination (species × flooding treatment × greenhouse) were extracted, root system washed and then scanned using WinRHIZO (Regent Instruments Inc., Canada) 35 days after the termination of the flooding treatment. The subsequent analysis gave total root length (cm), root surface area (cm^2), total projected area (cm^2), and volume (cm^3). To determine the presence of adventitious roots all 80 of the flooded tamarack were examined (40 flooded tamarack/CO_2 level). If the seedling showed new white roots growing on the soil surface at the end of the 28 day flooding period, it was recorded that they had adventitious roots. A seedling was determined to be dead if it was losing (or had lost all of) its needles, if the branches were brittle and if the needles did not show photosynthesis in the leaf chamber of the Portable Photosynthesis Machine. Mortality was tested for at the end of the recovery period.

The dry mass of foliage, stem and roots were measured after 16 hours of oven drying the biomass at 72 ^{o}C. To ensure that the biomass was in fact dry the weight of 10 root and 10 stem were measured every 2 hours starting 10 hours after drying began: when the weight was constant for two measurements the biomass was determined to be dry. The total biomass of the seedling was calculated by adding the dry weights of the stem, needles and roots. The root mass ratio was calculated by dividing the total root dry mass by the total seedling biomass. The leaf to root ratio

was calculated by dividing the total dry needle weight by the total dry root weight. The specific leaf area was calculated by dividing the leaf area by the total leaf dry mass. Finally the specific root length was calculated by dividing the total root length by the total dry root weight.

Data Analysis

The data was analyzed using a split-plot ANOVA, with CO_2 treatment at the whole plot level, and flooding treatment and species at the subplot level. Since both assumptions were met for all the variables, no data transformation was necessary. The full model used was:

$$Y_{ijkl} = C_i + \omega_{(i)j} + \delta_{(ij)} + F_k + CF_{jk} + \omega F_{(i)jk} + S_l + CS_{il} + \omega S_{(i)l} + CFS_{ikl} + \omega FS_{(i)jkl} + \varepsilon_{(ijkl)m}$$

where: i= 2 levels of CO_2, j= whole plot or greenhouses, k= 2 Flooding, l= 2 species, m= 4 subplot replicate, ω= whole plot error, δ= restriction due to randomization, S= Species, F= Flood Treatment, C= CO_2 level (Table 2-1.).

The assumptions of normality and homogeneity were assessed using the Shapiro tests and examining the residual plots respectively. When ANOVA showed a significant interaction, a Tukey's post-hoc test was used to compare the means. All the analyses were conducted using the "R" statistics software ("R", Geneva Switzerland).

To analyze the number of adventitious roots and percent mortality two different models were used due to the fact that these factors were measured as a count. For mortality a logistic regression with an identity link was used and a generalized linear model with a Poisson distribution and an identity link was used for adventitious roots. The model for mortality analysis:

$$Y_{ijkl} = C_i + F_j + CF_{ij} + S_k + CS_{ik} + CFS_{ijk} + FS_{jk} + \varepsilon_{(ijk)l}$$

where i= CO_2, j= Flooding, k= Species and l= Replicates.

Model for analyzing adventitious roots:

$$Y_{ij} = C_i + \varepsilon_{(i)j}$$

where i= CO_2 and j= Replication

Since adventitious roots only developed in flooded tamarack seedlings, species and flooding were not contained in the model.

Table 0-1. Expected mean square table for the full linear model.

	2 F i	2 R j	2 F k	2 F l		df
C_i	0	2	2	2	$\sigma^2 + 4\,\sigma^2\omega + 8\Phi C$	1
$\omega_{(i)j}$	1	1	2	2	$\sigma^2 + 4\,\sigma^2\omega$	2

$\delta_{(ij)}$	1	1	2	2	$\sigma^2 + 4\,\sigma^2\delta_{(ij)}$	0
F_k	2	2	0	2	$\sigma^2 + 2\,\sigma^2\omega F + 8\Phi F$	1
CF_{ik}	0	2	0	2	$\sigma^2 + 2\,\sigma^2\omega F + 4\Phi CF$	1
$\omega F_{(i)jk}$	1	1	0	2	$\sigma^2 + 2\,\sigma^2\omega F$	2
S_l	2	2	2	0	$\sigma^2 + 2\,\sigma^2\omega S + 8\Phi S$	1
CS_{il}	0	2	2	0	$\sigma^2 + 2\,\sigma^2\omega S + 4\Phi CS$	1
$\omega S_{(i)jl}$	1	1	2	0	$\sigma^2 + 2\,\sigma^2\omega S$	2
FS_{kl}	2	2	0	0	$\sigma^2 + \sigma^2\omega FS + 4\Phi FS$	1
CFS_{ikl}	0	2	0	0	$\sigma^2 + \sigma^2\omega FS + 2\Phi CFS$	1
$\omega FS_{(i)jkl}$	1	1	0	0	$\sigma^2 + \sigma^2\omega FS$	2
$\varepsilon_{(ijkl)}$	1	1	1	1	σ^2	0
						=63 df with m

Results

Doubled CO_2 significantly ($p = 0.004$, Table 2-2) increased the relative growth rate of height of both species (0.16 cm cm^{-1} vs. 0.46 cm cm^{-1}), while flooding or interactions among treatments had no significant effects on height RGR (Table 2-2). However, the flooding treatment significantly increased the specific leaf area (Flood average: 410.15 cm^2 g^{-1}, Control average: 104.92 cm^2 g^{-1}) (Table 2-2).

Neither CO_2 nor flooding nor their interactions had significant effects on the root collar diameter RGR (Table 2-2). However, it was significantly higher in *L. laricina* (0.32) than *P. mariana* (0.05 cm) (Table 2-2). Specific root length was significantly higher in the flooded seedlings (1539.33 cm g^{-1}) than the non-flooded (563.01 cm g^{-1}) (Table 2-2). The total root surface area was significantly decreased in flooded seedlings (Flood: 155.47 cm^2, Control: 370.22 cm^2) (Table 2-2).

Table 0-2. The P values for the effects of CO_2 concentration, flooding and species on the above ground and root characteristics in black spruce and tamarack seedlings. The titled columns are, H RGR= Height Relative Growth Rate, SLA= Specific Leaf Area, RC RGR= Root Collar Relative Growth Rate, SRL= Specific Root Length, SA= total root surface area and Root Vol= Root volume.

Terms	H RGR	SLA	RC RGR	SRL	Root SA	Root Vol
CO_2	**0.004**	0.277	0.288	0.383	0.358	0.426
Flooding	0.129	**0.009**	0.164	0.103	**0.006**	**0.003**
CO_2 × Flooding	0.444	0.788	0.207	0.866	0.291	0.419
Species	**0.044**	0.201	**0.027**	**0.003**	0.400	0.109
CO_2 × Species	0.458	0.714	0.401	0.144	0.809	0.896
Flooding × Species	0.413	0.403	**0.056**	0.715	0.381	0.351
CO_2 ×Flooding × Species	0.590	0.488	0.355	0.692	0.800	0.872

Flooded seedlings had significantly lower total biomass (3.88 g) than non-flooded (14.52 g) seedlings (Table 2-3). This was carried forward onto stem (Flood: 2.70 g, Control: 10.97 g), root (Flood: 0.83 g, Control: 3.02 g), and needle (Flood: 1.80 g, Control: 6.29 g) biomass which were all significantly lower in flooded than non-flooded seedlings (Table 2-3). Flooded

seedlings also had significantly higher leaf-root ratio (Flood: 3.65, Control: 2.21) and lower root mass ratios (Flood: 0.276, Control: 0.278) (Table 2-3).

Table 0-3. The effects (p values) of Doubled CO_2, Flooding and Species on the biomass (g) allocation of black spruce and tamarack seedlings.

Terms	Needle Mass	Root Mass	Total Mass	Leaf Root Ratio	Root Mass Ratio
CO_2	0.242	0.093	0.252	0.634	0.618
Flooding	**0.002**	**0.001**	**0.006**	0.149	**0.049**
CO_2 X Flooding	0.710	0.808	0.168	0.969	0.559
Species	0.652	**0.090**	0.731	**0.009**	0.161
CO_2 X Species	0.345	0.452	0.523	0.728	0.613
Flooding X Species	0.620	0.280	0.455	0.157	0.243
CO_2 X Flooding X Species	0.903	0.696	0.851	0.462	0.250

We observed that the doubled atmospheric CO_2 significantly increased the number of adventitious roots developed in flooded tamarack seedlings (Chi square= 8.47, $p<0.01$). Where ambient CO_2 *L. laricina* had 6 out of 32 seedlings had adventitious root and doubled CO_2 *L. laricina* had 17 out of 32 seedlings had adventitious roots. Mortality was significantly affected by CO_2, flooding and species. The mortality percentage of flooded of *P. mariana* seedlings in ambient CO_2 was 28%. The mortality percentage of flooded *P. mariana* seedlings in doubled CO_2 was 59%. There was no mortality for flooded *L. laricina* seedlings in ambient CO_2. The mortality percentage of flooded *L. laricina* in doubled CO_2 was 0.03%. There was no mortality in any of the non-flooded seedlings in either CO_2 level. Seedlings in doubled CO_2 had a significantly great mortality rate (Chi square= 7.58, $p<0.01$) and flooded seedlings had a higher

mortality rate than non-flooded seedlings (Chi-square= 50.60, $p<0.01$). Black spruce seedling had a mortality rate than tamarack seedlings give values (Chi-square=34.80, $p<0.01$).

Discussion

The morphological and biomass results did not support the hypothesis that doubled atmospheric CO_2 concentration could increase flood tolerance in *L. laricina* and *P. mariana*. None of the variables examined demonstrated a significant interaction between CO_2 concentration and flooding. The doubled CO_2 did increase the relative growth rate of seedling height. Surprisingly, this did not lead to increased stem biomass or total biomass, contrary to prior experimental evidence (Leakey et al. 2009). While a previous meta-analysis (Nie et al. 2013) concluded that increased atmospheric CO_2 would increase root growth and biomass accumulation, we found no indication that doubled atmospheric CO_2 had a positive effect on root growth or biomass accumulation with or without flooding. The lack of CO_2 effect on seedling's morphological response to flooding could be attributable to the short duration of the experiment. Morphological responses or acclimation generally take much longer time to show than physiological traits (Lambers et al. 2008). An alternative explanation could be that the physiological processes, such as photosynthesis, acclimated to the CO_2 regime of the treatment so that the actual effects of CO_2 elevation on the photosynthetic carbohydrate production was much less than assumed for the hypothesis. Indeed, photosynthetic down-regulation is commonly observed in CO_2 enrichment studies, particularly long term studies (Warren et al. 2015).

It was found that flooding reduced the total seedling biomass, as well as its components: stem, root, and needle biomass in both species: which is supported by previous literature (Islam and Macdonald 2004). This overall reduction in biomass was likely due to losses in root function and/or the amount of roots (Islam and Macdonald 2004, Grant et al. 2012), as

demonstrated by not only the reduced root biomass, but also the reduced root surface area, suggesting increased root mortality and/or reduced root growth. Flooded seedlings had a smaller total biomass than control seedlings and allocated more of their biomass to above ground structures, while the control allocated more to the root system. Allocating more biomass to above ground parts under flooded condition appears to be counterintuitive. However, the phenomenon could easily be a result of increased root mortality rather than a reduced biomass allocation to roots. The flooded seedlings allocated more biomass to above ground structures proportionally. Flooding did significantly increase specific leaf area. This was unexpected and was probably a result of reduced leaf mass rather increased leaf area. Indeed the leaf area of the flooded and control seedlings was comparable, while the control needle weight was significantly higher, leading to the control seedlings having the smaller specific leaf area. Since the foliage were already fully expanded at the initiation of the flood treatment and thus no structural or anatomic changes should have occurred during the flooding treatment, the impact of flooding on specific leaf area was most likely due to the loss of non-structural carbohydrates in the foliage of flooded seedlings. However, a question remains to be answered is whether longer term accumulation of CO_2 stimulated carbohydrate production would play a constructive role in the responses of seedlings to repeated occurrences of flooding.

There was little difference in morphological traits or biomass between the species in this experiment. Only the root collar relative growth rate was significantly different, with *L. laricina* developing a larger root collar than *P. mariana*, which is consistent with findings of previous studies (Montague and Givnish 1996). There was no significant interaction between species and atmospheric CO_2 concentration indicating that there will probably be no substantial changes in the relative competitiveness of the two species under the future climate conditions with elevated

atmospheric CO_2 concentration, at least in the seedling stage. The species also did not demonstrate a significant difference in their response to flooding either, despite the development of adventitious roots in some *L. laricina* seedlings in both ambient and doubled CO_2 concentration while *P. Mariana* did not produce any adventitious roots.

The limited number of significant interactions is likely due in part to the statistical model used. Split-plot ANOVAs tend to be conservative as they split errors into smaller groups than a completely randomized ANOVA. Though they are conservative in nature, a split-plot ANOVA was necessary for this experiment due to the experimental design. In order to have the proper numbers of seedlings for replication we used four greenhouses, which left us with two degrees of freedom in the whole plot error – significantly less than the 48 degrees of freedom left in the innermost residuals. It is also possible that these factors are additive and not interactive. Finally, this experiment used one-year old seedlings which may have meant they were more susceptible to such a drastic and high intensity flood. It is possible that only older and more resilient trees would be able to benefit from increased atmospheric CO_2. Future studies should focus on older trees to determine if their flood tolerance is affected by changes in atmospheric CO_2.

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Chapter 4: General Conclusion

This thesis demonstrated that *P. mariana* and *L. laricinia* reacted as expected to the main effects in the experiment. Seedlings in the doubled CO_2 significantly increased in relative height and demonstrated increased photosynthetic capacity and water-use efficiency in some of the measurements. Flooding significantly reduced total seedling biomass, stem biomass, root biomass, photosynthetic capacity, and root respiration. Further, flooded seedlings had decreased the maximum quantum yield of the Photosystem II in the photosynthetic apparatus from 22 days into the flood until the end of the experiment, indicating the full recovery of the photosynthetic function required an extended period of time following the flooding. There was no indication that the doubled atmospheric CO_2 altered either species' morphological response to flooding. However, there was some evidence that doubled atmospheric CO_2 reduced the seedlings stress levels six days into the flood. Seedlings had higher chlorophyll fluorescence when flooded in doubled CO_2 than in ambient CO_2 levels on the sixth day of the flood. Twenty-two days into the flood a significant difference in stress levels between species and flooding was observed. *P. mariana* had the highest chlorophyll fluorescence when not flooded, but the lowest when flooded. *L. laricinia* had the second highest chlorophyll fluorescence when not flooded and the second lowest when flooded. This is consistent with prior literature as *L. laricina* has been shown to have a higher flood tolerance than *P. mariana*. Finally, six days after flooding had ceased, photosynthetic rates were highest in non-flooded *L. laricina* followed by non-flooded *P. mariana* and then both species when flooded. This is consistent with previous literature which has shown *L. laricina* to have quicker growth rates than *P. mariana*. Overall, there was no consistent signal that doubled CO_2 could increase flooding tolerance in *L. laricina* and *P.*

mariana. This inconsistency could be due to the young age of the seedlings relative to the intensity of the flood or because these interactions are strictly additive. Further research is required to determine the actual relationship between these variables.